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Soil enzymatic responses to multiple environmental drivers in the Tibetan grasslands: Insights from two manipulative field experiments and a meta-analysis

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ABSTRACT

Soil enzymes are indicators of environmental changes, but uncertainties remain in predicting the impacts of environmental changes on soil enzyme activities in the cold environments. We integrated data from two manipulative field experiments and a meta-analysis in the alpine grasslands on the Tibetan Plateau. We examined the effects of multiple environmental drivers on the activities of soil extracellular enzymes involved in soil carbon (C)-, nitrogen (N)- and phosphorus (P)-cycling. The two manipulative field experiments showed that climate change (i.e., warming and altered precipitation) and nutrient (i.e., N and P) additions had no significant effects on the total, specific and ratios of soil C, N and P cycling enzyme activities in an alpine grassland ecosystem. The meta-analysis further showed no significant responses of soil enzyme activities to climate warming and nutrient additions across the Tibetan grasslands. Our findings indicate that soil extracellular enzymes are resistant to the major environmental changes in the Tibetan alpine grasslands with extreme environments. Thereby, accurate predictions of soil microbial responses to environmental changes require a thorough understanding of the soil microbial adaptation to the alpine ecosystems.

1. Introduction

Soil extracellular enzymes mediate nutrient cycling by catalyzing the biochemical reactions of decomposition. Work has shown that soil extracellular enzymes can be indicative of responses of ecosystem functions to global environmental changes (Burns et al., 2013; Henry, 2012; Sinsabaugh et al., 2008; Weedon et al., 2012). However, we do not have consistent observations on the effects of environmental changes on the activities of soil enzymes from the field experiments (see Allison and Treseder (2008); Jing et al. (2014); Keeler et al. (2008); Saiya-Cork et al. (2002)). Predictions about the impacts of warming and altered precipitation (Henry, 2012) and nutrient addition (Jian et al., 2016; Marklein and Houlton, 2012) on soil enzymes are challenging due to the complex and dynamic interactions among these multiple environmental drivers (Burns et al., 2013; Henry, 2012).

Abiotic factors, including soil temperature, together with soil moisture, may interact to drive the activities of soil extracellular enzymes. For instance, warming decreases the activity of chitin-degrading enzyme by decreasing soil moisture in boreal forest soils (Allison and Treseder, 2008). In contrast, warming enhances phenol oxidase activity when the water table depth was reduced in northern peatlands (Freeman et al., 2001). Furthermore, Henry (2012) found that the warming effects on both hydrolases and oxidative enzymes are less than the precipitation effects in the field experiments. He proposed that the magnitude and direction of soil enzymes in response to water manipulation partly depend on the variation of soil moisture among ecosystems. There is growing evidence suggesting that multi-factor climate change experiments are required to understand how soil enzymes respond to increasing environmental changes (Steinweg et al., 2013, 2012). However, few studies have examined the enzymatic responses to climate warming, altered precipitation patterns and their interactions simultaneously (but see Henry et al. (2005); Sardans et al. (2008); Steinweg et al. (2013)).

A broad consensus on the effects of nutrient additions on soil enzymes has been developed over decades. For instance, soil oxidative enzymes are often suppressed by N deposition, and are consistently

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considered as indicators of soil C sequestration in the forest ecosystems (Cusack et al., 2011, 2010; Eisenlord et al., 2013; Waldrop et al., 2004; Zak et al., 2008). In contrast, soil glycosidase enzymes (e.g., glucosidase, xylosidase and cellobiosidase) can be enhanced by N addition (Allison et al., 2008; Chen et al., 2017; Jian et al., 2016), while soil N-cycling enzymes (e.g., protein- and chitin-degrading enzymes) are negatively correlated with soil N availability (Shi, 2011) and are often suppressed by N addition (Allison et al., 2008). The same is true for soil P-cycling enzymes when P is added (Allison and Vitousek, 2005). However, knowledge of the influence of nutrient addition on soil enzymes in cold environments is currently inadequate (Chen et al., 2017; Jian et al., 2016), partially because the underlying mechanism is unclear (Nemergut et al., 2008).

The Tibetan Plateau has been experiencing dramatic environmental changes over the past decades (e.g., climate warming, altered rainfall regimes, N deposition and permafrost thawing) (Liu et al., 2013; Yu et al., 2012). These environmental changes are expected to have fundamental impacts on both above- and belowground biodiversity and ecosystem functioning (Jing et al., 2015; Yu et al., 2012). Meanwhile, the unique characteristics of the alpine grasslands (e.g., low temperature, large variation in temperature and rainfall, short growing season, locally adapted plant species, relatively young soils, etc.) provide a valuable opportunity to test whether soil enzymes are sensitive to environmental changes. However, the simultaneous effects of climate change and nutrient addition on soil enzymes have not been well documented in these alpine regions.

In this study, we used two multi-factor field experiments and a meta-analysis to simulate the major environmental changes including climate-change (warming and precipitation change) and nutrient-addition (N and P additions) in the Tibetan alpine grasslands. We measured the total (potential) activities of soil extracellular enzymes relevant to soil C-, N- and P cycling. We also standardized these total potential activities into the specific activities (per unit microbial biomass carbon) and calculated the ratios of soil C, N and P cycling enzyme activities. We predicted that warming and increased precipitation would enhance the activities of soil C, N and P cycling enzymes, while decreased precipitation would suppress the activities of these enzymes. N addition would suppress N-cycling and oxidative enzymes, but stimulate C- and P-cycling enzymes. P addition would suppress P-cycling enzymes, but increase C- and N-cycling enzymes. We expect that the meta-analysis would reveal whether the results from the two field experiments are generalizable across the whole alpine grasslands on the Tibetan Plateau.

2. Materials and methods

2.1. Manipulative field experiments

Our field research site is located at the Haibei Alpine Grassland Ecosystem Research (HAGER) station, Qinghai, China (Zhao and Zhou, 1999). HAGER station locates in a large valley of the Qilian mountain in the northeastern Tibetan Plateau. It has a typical and continental monsoon climate. The mean annual temperature is -1.1 °C and the mean annual precipitation is 485 mm (Table 1). The growing season (May – September) received *c.* 92–94% rainfall of the annual precipitation ranging from 331 to 461 mm, and the growing season mean air temperature ranged from 6.5 to 7.4 °C for the period of 2009–2012 (Wang et al., 2014). The vegetation type is the typical alpine grassland on the Tibetan Plateau. The dominant species are *Kobresia humilis*, *Elymus nutans* and *Stipa aliena*. The soil type is Mat-Cryic Cambisol (soil classification system of the Chinese National Soil Survey). It has a clay loam texture with an average thickness of 65 cm. More information on the characteristics of the research site and soil are shown in Table 1.

In 2011, two manipulative field experiments (i.e., climate-change and nutrient-addition) were conducted in the alpine grassland of the HAGER station. Experiment 1 (37°30′N, 101°12′E, 3200 m a.s.l.) is a climate-change experiment (i.e., warming and altered precipitation). Experiment 2 (37°37′N, 101°12′E, 3220 m a.s.l.) is a nutrient-addition experiment (i.e., N and P addition). The two manipulative field experiments have the same climate, vegetation and soil types (but differences are observed in soil available N and P) (Table 1). Thus they provide a unique opportunity to examine the effects of multiple environmental factors on soil processes (i.e., enzymes in this study). Note that the interactions of climate-change and nutrient-addition field experiment were not conducted due to logistical limitations and cost of field experiments. We showed the landscape photos of the two field experiments in Fig. S1.

Table 1
Site and soil characteristics of the climate-change (Experiment 1) and nutrient-addition (Experiment 2) experiments.

	Experiment 1	Experiment 2
Latitude	37°30′ N	37°37′ N
Longitude	101°12′ E	101°12′ E
Altitude (m)	3200	3220
[a]Mean annual temperature (°C)	−1.1	−1.1
[a]Mean annual precipitation (mm)	485	485
Vegetation type	Alpine grassland	Alpine grassland
Dominant species	*Kobresia humilis, Elymus nutans, Stipa aliena*	*Kobresia humilis, Elymus nutans, Stipa aliena*
[b]Soil type	Mat-Cryic Cambisols	Mat-Cryic Cambisols
Soil texture	Clay loam	Clay loam
[c]Soil pH	8.01 ± 0.06	7.50 ± 0.01
Soil total C (g/kg)	78.2 ± 0.95	75.7 ± 1.4
Soil organic C (g/kg)	63.1 ± 1.0	68.6 ± 1.6
Soil total N (g/kg)	5.8 ± 0.3	5.5 ± 0.3
Soil NH_4-N (mg/kg)	27.3 ± 4.2	3.7 ± 0.1
Soil NO_3-N (mg/kg)	10.3 ± 0.5	22.3 ± 1.1
[d]Soil available P (mg/kg)	7.7 ± 0.2	17.5 ± 1.1

[a] Climate data are compiled from the same weather station for the two experimental sites.

[b] Soil type is classified according to the classification system of the Chinese National Soil Survey.

[c] Values of soil variables and the follows are mean (n = 4 [Experiment 1] or 5 [Experiment 2]) ± 1SE at 0–10 cm soil depth.

[d] Soil available P was measured as Olsen (1954).

Experiment 1 is a two-way factorial climate-change experiment with a randomized block design. The two main factors are warming (two levels: T0 and T+) and altered precipitation (three levels: 0, -50%, +50%) with six treatment combinations including T0P0, T0P-, T0P+, T + P0, T + P- and T + P + . A total of 36 plots (1.8 m in width × 2.2 m in length with 2.5 m buffer) within six blocks were established in the early growing season of 2011. Overhead infrared heaters were used to simulate climate warming in the alpine grassland ecosystem. Warming increased soil temperature by ~ 1.6 °C and decreased soil moisture by ~3% at 5 cm depth (Lin et al., 2016). Such increase in soil temperature approximates the climate warming over the past 50 years (~ 0.3 °C increase in air temperature per decade) on the Tibetan Plateau (Chen et al., 2013). To manipulate precipitation, rainout shelters were used to intercept 50% of rainfall for the 50% reduction in precipitation. The intercepted rainfall was collected and evenly sprayed onto the plots with a 50% increase in precipitation. We used the 50% reduction or increase in precipitation because a 50-year observation showed a similar magnitude of interannual variation in annual precipitation in this area (Chen et al., 2013). Precipitation treatments had no effects on soil temperature, and soil moisture was decreased to 23% by -50% precipitation treatment, while was increased up to 29% by +50% precipitation treatment (Lin et al., 2016). See Xu et al. (2018) for more information on the experimental design of the climate-change experiment.

Experiment 2 is a randomized block design with the applications of urea addition (four levels: 0, 25, 50 and 100 kg N ha^{-1} yr^{-1}) and P

addition (superphosphate, 0 and 50 kg P ha^{-1} yr^{-1}) including six treatment levels (i.e., N0, N25, N50, N100, P50 and N100P50). We used this combination of N and P treatments because experiment 2 allows us to isolate the effects of the rate of N addition (i.e., N0, N25, N50 and N100) and the interactions of N and P additions (i.e., N0, N100, P50 and N100P50) on soil enzymes. A total of 36 plots (6 m × 6 m) within six blocks were established in the growing season of 2011. Urea and superphosphate were manually added three times during the growing season at each year (i.e., June, July and August). The addition dates were June 5, July 1 and August 1 in 2015. Soil samples were taken ~ two weeks following the last nutrient addition. The rate of N addition in this study is greater than the natural N deposition which ranges from 10 to 15 kg N ha^{-1} yr^{-1} (Jia et al., 2014). In addition, Jiang et al. (2012) found that the energy cost of plant N uptake from soils is greater than P indicating N is limited in the alpine grassland ecosystem we studied. Thus, we expected that the alpine grassland ecosystem would respond more strongly to the high concentrations of N addition (e.g., 21% increase of the aboveground net primary production (Ren et al., 2017)). However, we did not have specific predictions on the sign and magnitude of P addition given that our study regions are assumed to be N- but not P-limited (Jiang et al., 2012; Ren et al., 2017).

2.2. Sample collection and laboratory measurements

We collected soil samples in August 2015 (the peak growing season of the alpine grassland and four years after starting the field manipulative experiments). Three soil cores (5 cm in diameter) were randomly taken at 0–10 cm soil depth at each plot. The surface litter was removed before soil sampling. The three soil samples were pooled in the field, sieved (2 mm) and stored at -20 °C for further analysis. Due to logistical limitations, we used only subset samples of the climate-change and nutrient-addition experiments. In total, we collected 24 samples from experiment 1 (four blocks × six treatments per block) and 30 samples from experiment 2 (five blocks × six treatments per block). For experiment 1, warming treatment has two levels and 12 samples per level. Precipitation treatment has three levels and eight samples per level. For experiment 2, N addition has four levels and the sample sizes for each level are 10 (N0), 5 (N25), 5 (N50) and 10 (N100). P addition treatment has two levels and the sample sizes for each level are 20 (P0) and 10 (P50).

We measured the potential activities of seven soil extracellular enzymes, including five hydrolytic enzymes (β-1,4-glucosidase (BG), β-D-cellobiohydrolase (CB), β-1,4-*N*-acetylglucosaminnidase (NAG), L-leucine aminopeptidase (LAP) and acid phosphatase (AP)) and two oxidative enzymes (phenol oxidase (POX) and peroxidase (PER)). The activities of these extracellular enzymes were assayed based on the methods described by German et al. (2011); Bach et al. (2013) and Jing et al. (2017) (see Appendix A for more information of the enzyme assays). Note that the assay temperature used in this study is based on the temperature optima of soil enzymes, which can reach up to 27 °C or over 30 °C in our system (Jing et al., 2014). In addition, we measured soil microbial biomass carbon to calculate the specific enzyme activity. We used the chloroform fumigation-extraction method to measure soil microbial biomass carbon (Vance et al., 1987) (See Appendix B for a brief introduction to this method).

We calculated the total, specific and the ratios of C, N and P cycling enzyme activities. The total enzyme activity is a measure of the potential enzyme activity. It is not the actual enzyme activity measured *in situ*, but indicates the overall enzyme concentrations (Wallenstein and Weintraub, 2008). The specific enzyme activity is an indicator of the changes in microbial resource allocation patterns that is generally normalized to the size of microbial biomass or soil carbon. Thus it can be used to make comparisons among soils with different microbial biomass or carbon contents (Stone et al., 2014; Trasar-Cepeda et al., 2008; Weintraub et al., 2013). The ratios of C, N and P cycling enzyme activities are measures of microbial allocation to energy versus nutrient acquisition (Sinsabaugh et al., 2009). Specifically, the total enzyme activity, the conversion rate of the enzymatic substrate, was calculated as described by German et al. (2011). It was expressed as nmol g dry $weight^{-1}$ h^{-1} for hydrolytic enzymes and μmol g dry $weight^{-1}$ h^{-1} for oxidative enzymes. We then calculated the specific enzyme activity by normalizing the total enzyme activity as per unit of soil microbial biomass carbon (Stone et al., 2014). Finally, we classified the four hydrolytic enzymes (BG, NAG, LAP and AP) into three functional groups including C- (BG), N- (NAG and LAP) and P- (AP) cycling enzymes (Sinsabaugh and Follstad Shah, 2012; Sinsabaugh et al., 2008). We calculated the ratios of C:N:P cycling enzymes as ln(BG) / ln(AP), ln (BG) / ln(NAG + LAP) and ln(NAG + LAP) / ln(AP), respectively.

2.3. Data analyses

To examine the effects of environmental changes on the total, specific and the ratios of enzyme activities related to C, N and P cycling and soil microbial biomass C, we performed the linear mixed-effect models using lme4 and lmerTest packages (Bates et al., 2015; Kuznetsova et al., 2016). Type I Analysis of Variance with Kenward-Roger approximation for degrees of freedom was used for the analyses of the linear mixed-effect models. The total, specific and the ratios of C, N and P cycling enzyme activity were treated as response variables. Block was treated as random factors. For experiment 1, we treated warming (two levels), precipitation (three levels) and their interactions as fixed factors. For experiment 2, the experimental design allowed us to test the effects of nutrient additions on soil enzymes in two ways. We used subset data from experiment 2 to perform the analyses. First, we treated a linear contrast of N addition and the deviation from the linear contrast of N addition as the fixed factors. Second, we treated N and P additions and their interactions as fixed factors. Note that the experimental design did not allow us to test the interactions between climate change and nutrient addition.

To examine the effects of environmental changes on the magnitude and direction of soil enzyme activities, we calculated the relative changes of the total (potential), specific and the ratios of enzyme activities. Relative change (%) = [(treated - control) / control * 100]. Treated were values from the treatment applied plots (warming, -50% and +50% precipitation, N and P additions) and control were values from the plots without any applications of the warming and precipitation treatments or without N and P additions. We further extracted the 95% confidence interval of the relative changes of each soil enzyme activity based on the Student's t-Test. The significance test of the relative changes was based on the 95% confidence interval. An overlap of the error bars (95% confidence interval) was considered as a lack of treatment effect on soil enzyme activities.

To further examine whether our findings are consistent with the other experimental studies on the effects of environmental changes on soil enzyme activity in the Tibetan alpine grasslands, we searched papers published in English and/or in Chinese from the Web of Science and the China National Knowledge Infrastructure (CNKI) (http://www.cnki.net/) with no restriction on publication year on September 20, 2017. We used the keywords "soil" AND "enzyme" AND "Tibet*" NOT "forest". A total of 54 papers in Web of Science and 69 papers in CNKI were founded. We excluded 112 papers that were relevant to laboratory incubations, non-grassland ecosystems (e.g., agricultural and wetland ecosystems) and reviews. If sample sizes, standard deviations or standard errors were not reported, these observations were not selected. We used only data that reported at the latest sampling dates if soil enzymes were measured more than once. We included only 11 papers related to the manipulative field experiments that temperature, precipitation and nutrient additions (i.e., N and P) were the main factors in the Tibetan alpine grasslands. We further checked the references in case relevant work was missed. A total of 11 papers with 86 observations were compiled (Appendix C) using GetData Graph Digitizer (http://getdata-graph-digitizer.com/). Among all the papers we compiled, we did not

find any data from precipitation experiments. Thus, we analyzed only data from the field experiments of climate warming, N and P additions. Because the sample sizes were small, we classified soil enzymes into three functional groups (i.e., C-, N- and P-cycling enzymes) as above. Due to the methodological differences of enzyme assays among studies, there were not enough data for BG compiled from previous work. Consequently, we used all C acquiring enzymes to quantify the activity of soil C-cycling enzymes. In addition, the meta-analysis did not allow us to quantify the relative change of enzyme activities because the raw data were not available for most studies. The effect size of logarithm response ratio and 95% confidence intervals were calculated. We considered an overlap of zero as non-significant treatment effects. All data analyses in this study were performed in R 3.3.3 (R Development Core Team, 2016). The meta-data and R scripts have been deposited to Github (https://github.com/XJingPKU/TibetEnzyme).

3. Results

3.1. Effect of climate change on soil enzymes

Warming had no significant effects on the total, specific and ratios of C, N and P cycling enzyme activities in most cases (Fig. 1, S1 and S2), except that warming increased the specific activity of AP from 0.49 ± 0.03 to 0.58 ± 0.04 (hereafter mean ± 1se, $n = 12$) nmol g dry weight^{-1} h^{-1} / mg C kg^{-1} ($F = 7.89$, $P = 0.013$, Table 2 and 3). We observed that decreasing precipitation (i.e., -50%) only decreased the total activity of AP from 317 ± 31 to 279 ± 24 ($n = 8$) nmol g dry weight^{-1} h^{-1} ($F = 5.13$, $P = 0.020$, Table 2 and 3). Increasing precipitation (i.e., +50%) increased the total activity of POX from 378 ± 29 to 492 ± 22 ($n = 8$) μmol g dry weight^{-1} h^{-1} ($F = 6.28$, $P = 0.10$, Table 2 and 3), while decreased the specific activity of PER from 0.50 ± 0.07 to 0.29 ± 0.02 ($n = 8$) μmol g dry weight^{-1} h^{-1} / mg C kg^{-1} ($F = 6.84$, $P = 0.010$, Table S1). Additionally, warming with decreasing precipitation treatment (T+P-) increased the specific activity of AP (17%, $P = 0.048$, Table S2). Furthermore, we found 50% decrease in precipitation decreased microbial biomass carbon from 582 ± 27 to 503 ± 14 mg kg^{-1} ($n = 8$, Table 3 and S2) There were no interactions between warming and precipitation on the total, specific and C, N and P cycling enzyme activities ($P > 0.05$, Table 2).

3.2. Effects of nutrient additions on soil enzymes

N addition (i.e., linear N contrast and the deviation of linear N contrast) had no significant effects on the total, specific and ratios of C, N and P cycling enzyme activities ($P \geq 0.05$, Table 2 and S1). In addition, P addition increased the total activity of PER from 203 ± 17 ($n = 20$) to 302 ± 21 ($n = 10$) μmol g dry weight^{-1} h^{-1} ($F = 5.66$, $P = 0.035$, Table 2 and 3), while decreased the specific activity of BG from 0.46 ± 0.05 to 0.34 ± 0.05 nmol g dry weight^{-1} h^{-1} / mg C kg^{-1} ($F = 4.98$, $P = 0.049$) and that of AP from 0.74 ± 0.09 to 0.56 ± 0.05 nmol g dry weight^{-1} h^{-1} / mg C kg^{-1} ($F = 5.31$, $P = 0.042$, Table S1). There were no interactions between N and P additions on the total, specific and C, N and P cycling enzyme activities ($P > 0.05$, Table 2 and S1). We did not find any significant effect of nutrient addition on soil microbial biomass C or N (Table S2).

Specifically, we did not observe significant changes in soil total enzyme activity when nutrients were added (Fig. S1), except that low-N addition (25 kg N ha^{-1} yr^{-1}) and P addition decreased BG activity by 25% ($P = 0.009$) and 31% ($P = 0.025$), respectively, and high-N addition (100 kg N ha^{-1} yr^{-1}) decreased peroxidase activity by 23% ($P = 0.034$, Table 3 and S2). Additionally, nutrient addition had no effect on the specific activity of soil enzymes (Fig. S1), except that the medium-N addition (50 kg N ha^{-1} yr^{-1}) decreased the specific activities of BG (42%, $P = 0.027$, Table S1) and CB (51%, $P = 0.009$, Table S1). Finally, N and P addition had no effect on the ratios of soil enzyme activities (Fig. S2).

3.3. Meta-analysis

The meta-analysis showed that warming had no significant effects on the total activity of C-, N- and P-cycling enzymes in the Tibetan grasslands (Fig. 2). Further, there were no significant effects of N and P addition on these three functional groups of soil enzymes (Fig. 2). Finally, we found that the combined additions of N and P weakly decreased C-cycling enzymes, weakly increased N-cycling enzymes, but had no effect on P-cycling enzymes (Fig. 2). The lack of measurements

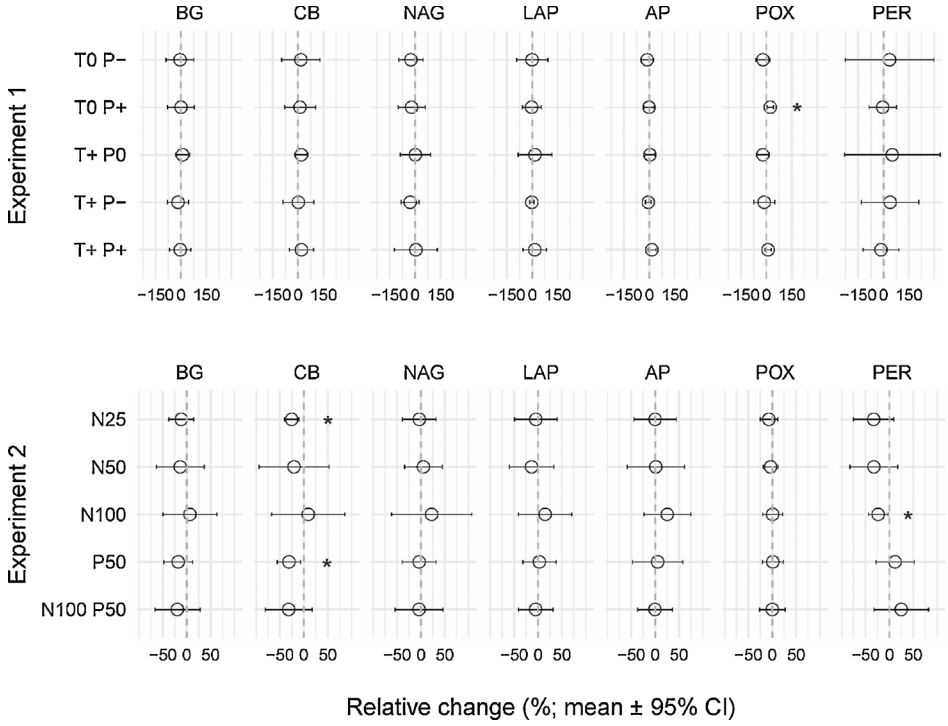

Fig. 1. Percentage change of the total activities of soil enzymes in the climate-change and nutrient-addition experiments. Relative change (%) = [(treated - control) / control * 100]. Error bars show 95% confidence interval. The sample size is 4 for the climate-change experiment and 5 for the nutrient-addition experiment. * represents significant changes in soil enzyme activities at $P < 0.05$. BG = β-1,4-glucosidase, CB = β-D-cellobiohydrolase, NAG = β-1,4-N-acetylglucosaminnidase, LAP = L-leucine aminopeptidase, AP = acid phosphatase, POX = phenol oxidase, PER = peroxidase.

Table 2
Summary of the linear-mixed effect models for the two manipulative field experiments. W, warming; P, precipitation; W:P, interaction between warming and precipitation; N.linear, linear contrast of N addition; N.factor, deviation of N additioin; N, N addition; P, P addition; N:P, interaction between N and P additions. BG, β-1,4-glucosidase; CB, β-D-cellobiohydrolase; NAG, β-1,4-*N*-acetylglucosaminnidase; LAP, L-leucine aminopeptidase; AP, acid phosphatase; POX, phenol oxidase; PER, peroxidase. Significant treatment effects ($P < 0.05$) are marked in bold.

	Experiment 1			Experiment 2					
Enzymes	Sources	*F*	*P*	Sources	*F*	*P*	Sources	*F*	*P*
BG	W	0.05	0.829	N.linear	0.03	0.855	N	0.18	0.680
BG	P	2.27	0.138	N.factor	1.85	0.200	P	1.99	0.184
BG	W:P	0.07	0.934				N:P	0.14	0.712
CB	W	1.09	0.313	N.linear	0.03	0.860	N	0.28	0.608
CB	P	0.35	0.708	N.factor	3.90	0.050	P	4.50	0.055
CB	W:P	0.24	0.791				N:P	0.10	0.756
NAG	W	0.80	0.386	N.linear	0.09	0.766	N	0.00	0.950
NAG	P	1.80	0.200	N.factor	0.02	0.976	P	1.19	0.297
NAG	W:P	1.20	0.328				N:P	0.21	0.654
LAP	W	2.08	0.170	N.linear	0.47	0.506	N	0.02	0.891
LAP	P	0.63	0.545	N.factor	1.97	0.182	P	0.55	0.472
LAP	W:P	0.37	0.695				N:P	0.85	0.376
AP	W	**5.58**	**0.032**	N.linear	3.24	0.097	N	0.76	0.399
AP	P	**5.13**	**0.020**	N.factor	1.34	0.299	P	2.14	0.169
AP	W:P	1.04	0.376				N:P	1.64	0.224
POX	W	1.48	0.242	N.linear	0.12	0.732	N	0.06	0.813
POX	P	**6.28**	**0.010**	N.factor	1.02	0.391	P	0.00	0.960
POX	W:P	1.06	0.371				N:P	0.05	0.827
PER	W	1.68	0.219	N.linear	1.31	0.274	N	0.67	0.431
PER	P	3.02	0.085	N.factor	2.77	0.102	P	**5.66**	**0.035**
PER	W:P	0.91	0.428				N:P	2.53	0.138

(or reports) on microbial biomass and all relevant hydrolytic enzymes in these studies prevented us from calculating specific enzyme activities and enzyme stoichiometric ratios.

4. Discussion

In this study, we combined two manipulative field experiments and a meta-analysis to examine the responses of soil extracellular enzymes to major environmental changes in the Tibetan alpine grasslands. Our results showed that climate warming, altered precipitation, and nutrient addition had no consistent effects on soil enzyme activities (i.e., total, specific and ratios of the C, N and P cycling enzymes) in most cases. Moreover, we did not find interactions between climate warming and altered precipitation and between N addition and P addition, and the rate of N addition had no effect on soil enzyme activities. These findings were further supported by the meta-analysis that climate warming, N and P additions had no significant effects on the three functional groups of soil enzymes (C, N and P acquisition). Collectively, these results did not support our initial predictions. For instance, climate warming and increased precipitation did not enhance soil enzyme activities, while decreased precipitation did not suppress soil enzyme activities, no matter what enzyme activities were examined (i.e., the total activities, the specific activities and the ratios of C-, N- and P cycling enzymes). Furthermore, our findings did not support the resource allocation theory, which predicts that the activities of soil microbial enzymes involved in C-, N- and P-cycling are mediated by microbial substrate availability (Allison and Vitousek, 2005; Sinsabaugh and Moorhead, 1994; Sinsabaugh et al., 2008). Taken together, our findings suggest that soil extracellular enzymes are not sensitive to the major environmental changes in the Tibetan alpine grasslands.

4.1. Effects of warming and altered precipitation

We found that climate warming with decreasing precipitation treatment enhanced only the specific activity of AP that probably resulted from the stimulated microbial production of phosphatase relative

Table 3
Soil enzyme activities and microbial biomass crabon in the climate-change and nutrient-addition experiments. The unit is nmol/g soil d.w./h for BG, CB, NAG, LAP and AP, μmol/g soil d.w./h for POX and PER and mg C/kg soil d.w. for microbial biomass carbon (MBC). Data shown are mean ($n = 4$) ± 1SE for experiment 1 (climate-change experiment) and mean ($n = 5$) ± 1SE for experiment 2 (nutrient-addition experiment). In experiment 1, warming treatment has two levels (T0 and T+) and precipitation treatment has three levels (P0, P- and P+). In experiment 2, N addition has four levels (N0, N25, N50 and N100) and P addition has two levels (P0 and P50). BG, β-1,4-glucosidase; CB, β-D-cellobiohydrolase; NAG, β-1,4-N-acetylglucosaminnidase; LAP, L-leucine aminopeptidase; AP, acid phosphatase; POX, phenol oxidase; PER, peroxidase.

	Treatments	BG	CB	NAG	LAP	AP	POX	PER	MBC
Experiment 1	T0 P0	219.6 ± 88.7	58.5 ± 26.8	14.0 ± 5.3	172.8 ± 49.5	313.4 ± 69.3	424.5 ± 44.3	228.7 ± 47.7	607.4 ± 48.7
	T0 P-	168.3 ± 26.2	53.8 ± 13.1	8.4 ± 1.0	147.7 ± 21.6	262.0 ± 32.4	340.0 ± 77.0	270.0 ± 66.5	521.5 ± 7.1
	T0 P+	174.8 ± 11.8	50.7 ± 7.3	10.3 ± 4.4	153.1 ± 20.9	300.9 ± 51.6	520.7 ± 50.1	204.4 ± 3.4	675.4 ± 14.6
	T + P0	225.6 ± 74.7	68.2 ± 28.8	12.1 ± 3.4	179.3 ± 35.8	320.3 ± 63.4	331.7 ± 56.3	320.4 ± 77.4	556.0 ± 23.7
	T + P-	162.5 ± 56.3	55.2 ± 26.8	10.9 ± 6.3	166.6 ± 41.6	295.1 ± 64.7	365.7 ± 108.5	327.8 ± 64.8	483.7 ± 25.3
	T + P+	189.4 ± 58.4	66.5 ± 25.8	13.7 ± 6.6	189.7 ± 53.9	354.2 ± 58.7	463.1 ± 29.6	176.9 ± 34.8	635.0 ± 7.6
Experiment 2	N0 P0	252.7 ± 55.2	57.1 ± 15.6	16.5 ± 4.3	161.6 ± 25.1	359.4 ± 61.0	566.9 ± 35.3	268.9 ± 48.0	513.3 ± 122.9
	N25	213.0 ± 24.9	41.7 ± 10.4	16.0 ± 6.0	148.8 ± 30.4	335.6 ± 31.9	523.9 ± 40.9	171.8 ± 57.1	570.3 ± 99.9
	N50	200.0 ± 33.1	38.4 ± 9.6	16.6 ± 3.8	130.1 ± 20.3	333.7 ± 38.5	544.4 ± 38.5	168.7 ± 53.2	714.0 ± 67.3
	N100	251.2 ± 43.6	55.4 ± 11.3	17.3 ± 4.0	175.1 ± 23.0	431.6 ± 66.7	566.2 ± 38.3	201.3 ± 32.7	547.1 ± 98.6
	P50	217.2 ± 85.7	42.7 ± 20.6	15.1 ± 3.4	164.7 ± 40.3	353.3 ± 41.2	576.8 ± 86.6	291.1 ± 47.6	583.9 ± 47.8
	N100 P50	189.6 ± 54.8	36.0 ± 14.1	13.9 ± 1.4	146.4 ± 16.0	339.7 ± 36.8	560.1 ± 52.4	312.8 ± 47.4	738.3 ± 107.0

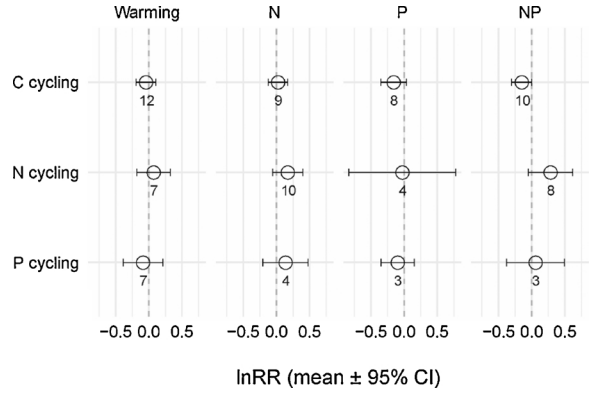

Fig. 2. Meta-analysis on the effects of warming, N and P additions on soil C-, N- and P-cycling enzymes in the alpine grasslands on the Tibetan Plateau. Points represent the effect size [ln(treatment / control)] of warming, N and P additions. Error bars show 95% confidence interval. The number under each point is the sample size for each treatment.

to microbial biomass C. The increased specific activity of AP was not in agreement with an observation that warming decreased organic and total P at 0–10 cm soil depth, while increased the activities of acid- and alkaline-phosphomonoesterase in the alpine grassland (Rui et al., 2012). The decreased microbial biomass C due to the reduced precipitation treatment may contribute to the increased specific activity of AP observed because we did not find any significant effect of warming with decreasing precipitation treatment on the total activity of AP. In contrast to P cycling enzyme, our climate change experiments and the meta-analysis did not show a significant warming effect on the total activity of C and N cycling enzymes. The low magnitude of warming effects on soils (1–2 °C) in all of these studies may partially explain the lack of response of soil enzymes to warming treatment. Though unexpected, this supported the findings of earlier studies at our site, where warming had no effects on the activity and temperature sensitivity of four glycosidases and phenol oxidase (Jing et al., 2014). Furthermore, although the forage quality (i.e., crude protein, hemicellulose, cellulose, lignin and digestibility) of plant community were altered after three- to five-year warming treatment (Xu et al., 2018), there were no changes in most of the soil N pools (e.g., total N, ammonium, dissolved organic N and amino acid) and soil microbial community diversity and composition in the early stage (2013, two years after treatment) of the warming experiment (Lin et al., 2016; Zhang et al., 2016). Collectively, these findings indicated that soil microbial communities did not show significant responses to short-term climate change in this alpine grassland.

In contrast to warming, it has been reported that altered precipitation patterns generally have a greater influence on soil enzyme activities than warming treatment in the field (Henry, 2012). Our findings partially supported this finding. We observed that -50% precipitation decreased the total activity of AP, but +50% precipitation increased the total activity of POX and decreased the specific activity of PER. However, these effects were absent for the other enzymes. According to a synthesis (Henry, 2012), the activities of soil enzymes generally vary with the gradients of soil moisture among ecosystems. For instance, enzyme activities can be stimulated in dry soils, have no responses in well-drained soils, but are reduced in water-logged soils. In our study, a few enzymes responded to the altered precipitation treatment, yet there were no evidence of shifts in the activities of soil enzymes in total, probably because the manipulations of precipitation (± 50%) were not large enough to substantially reduce or increase soil moisture to its extreme. Note that our research site is a mesic grassland, the 50% reduction and increase in precipitation are within the interannual variations in annual precipitation at this site (Chen et al., 2013), and may not lead to extreme soil moisture. For instance, Lin et al. (2016) found that 50% precipitation addition increased soil moisture up to 29% (v/v) that is close to the water holding capacity at this site. In addition, soil fungi are important producers of soil extracellular enzymes (Allison and Treseder, 2008) and a previous study found that precipitation treatments had no significant effect on soil fungal communities at our site (Zhang et al., 2016). Thus, such weak responses in soil fungal communities could partially explain our findings that most of the soil enzymes did not respond to the altered precipitation treatments in the alpine grassland.

4.2. Effects of nitrogen and phosphorus additions

Both our manipulative field experiment and meta-analysis showed that N addition had no effects on these enzymes examined. This finding was consistent with an earlier observation at our site which had found a neutral effect of N addition on the activities of four glycosidases, NAG, AP and POX in the topsoil after a two-year field experiment (Jing et al., 2016). Furthermore, a recent study from the same alpine grassland showed that N addition had no effects on belowground biomass, soil dissolved organic carbon, soil total N, ammonium, pH, and soil moisture and soil fungal diversity and community composition (He et al., 2016). Interestingly, He et al. (2016) found that there were no differences in soil available N (58.9 ± 6.3 mg kg^{-1} in control plots and 58.8 ± 6.0 mg kg^{-1} in N added plots), suggesting N uptake by plants or N loss through leaching and volatilization were high in the alpine grassland. Chen et al. (unpublished data) using the same soil samples as in this study found that N addition had no effect on soil microbial biomass N (Table S2). Thus, we speculated that the high N use efficiency (or N loss) and no changes in soil N availability and soil microbial biomass N could contribute to the no responses of soil enzymes to N addition. In addition, Jing et al. (2016) pointed out that the short period of the experiment, moderate rate of N addition and minor responses of root biomass, soil microbial biomass and soil respiration could explain the lack of N addition effect on soil enzymes in the alpine grassland. Our finding in the current study provided new evidence that both the four-year field experiment and the rate of N addition (e.g., 25–100 kg N ha^{-1} yr^{-1}) had no significant effects on soil enzymes.

Despite the general lack of responses of soil enzymes to N addition in the alpine grassland, we found a few cases that were consistent with studies in other ecosystems if we took a look at the relative changes of soil enzymes. For instance, high-N addition (N100) suppressed peroxidase activity, which has been consistently reported in forest ecosystems (Zak et al., 2008). Furthermore, the decreased peroxidase activity due to N addition may further slow the decomposition rate of soil organic matter, and finally increase soil C storage in the long run (Zak et al., 2008).

This study did not reveal that the production of C and N cycling enzymes depended on the availability of P for enzyme production. By adding P, the biomass and abundance of (N fixing) legume plants and soil available P were significantly increased in this alpine grassland, while the aboveground net primary production did not change (Ren et al., 2017), thereby we expected that carbon would become limiting factor relative to available N and P, and microbes would produce C cycling enzymes to acquire C. However, our findings only partially supported this prediction. We found that P addition increased the total activity of PER. In contrast, we found that P addition decreased the specific activity of BG and AP. The relative changes in CB activity also decreased when P was added. These findings were consistent with a previous finding that P addition suppressed a suite of hydrolytic enzymes in topsoil in the alpine grassland ecosystem (Jing et al., 2016). The decreased soil fungal diversity and the abundance of Glomeromycetes (mycorrhizal fungi acquiring *c.* 5–20% N and 75% P for plants) (He et al., 2016) might result in the decline of soil enzyme activities with P addition. In addition, we did not find interactions between N and P additions on soil enzyme activities. This could be caused by the weak effects of N and P additions on soil enzyme activities.

4.3. Limitations and implications

Soil enzymatic responses could be caused by both direct and indirect treatment effects, which make it difficult to pinpoint the exact mechanisms to link the responses of soil enzyme activities to environmental changes (Henry, 2012; Nannipieri et al., 2012). For instance, soil microbes might adapt to the alpine climate (Saavedra et al., 2018) that is characterized by high variations in temperature diurnally and seasonally and/or short growing season (Jing et al., 2014; Wang et al., 2014). In this study, we examined only the apparent effects of climate change and nutrient addition on soil extracellular enzymes, while we cannot rule out the direct vs. indirect effects of these multiple environmental drivers on soil microbial activities through changes in environment, soil and plant characteristics. Because there were no studies reporting the effect of precipitation on soil enzymes in our meta-analysis, it was difficult to generalize our findings about the influence of precipitation on soil enzymes across the Tibetan grasslands. Another limitation was that our studies did not consider the temporal (seasonal) dynamics of soil enzyme activities (Baldrian et al., 2013; Bell et al., 2010) in response to the major environmental changes in the alpine grasslands. Although there are few studies reporting the long-term multiple environmental drivers on soil enzymes, the duration of a treatment can determine the enzymatic responses to nutrient additions (Ajwa et al., 1999; Allison et al., 2010; Chen et al., 2017; Wang et al., 2015; Xiao et al., 2018). For instance, a greenhouse experiment showed that short-term (~ 165 days) inorganic N addition had a weak effect on soil enzymes, while soil enzyme activity was decreased after 304 days of inorganic N application (Fauci and Dick, 1994). In a field experiment, Bell et al. (2010) failed to detect any significant enzymatic responses to N addition and climate warming with seasonal dynamics. However, it has been reported that soil glycosidase activity can be enhanced while oxidases can be depressed by long-term application of N addition (Xiao et al., 2018).

Our study did not find that climate change and nutrient addition influenced the ratios of C, N and P cycling enzymes. This finding was not consistent with a global scale study that the ratio of C:P cycling enzymes was negatively related to mean annual temperature and precipitation, and the ratio of C:N cycling enzymes was positively related to mean annual precipitation (Sinsabaugh et al., 2008). At the global scale, environmental gradients apparently play an important role in the spatial distribution of soil enzymes. For instance, climate can indirectly affect soil pH through changes in soil weathering and plant community composition. The changes in soil pH could further mediate soil microbial activities of C, N and P acquisition through changes in soil nutrient availability, organic matter content and microbial community composition (Sinsabaugh et al., 2008). However, we did not observe significant and generalizable responses of soil enzymatic stoichiometry to climate change and nutrient addition in this study. Environmental gradients generated by climate change and nutrient addition may be not large enough to affect the optima of soil enzymes (e.g., pH and temperature) at our study site. Additional factors which are not assessed in this study may contribute to our observations, such as seasonal dynamics of soil enzymes, plant and soil interactions and cold adaptation of soil microbes. Alternatively, the lack of a shift of soil enzymatic stoichiometry was due to the lack of responses of soil enzymes individually in this study.

Despite these uncertainties, our findings indicate that the contemporary environmental changes and their interactions cannot predict soil microbial activities regarding the total, specific or ratios of soil enzyme activities in the alpine grassland ecosystems. Although these findings are out of our expectation, soil extracellular enzymes are apparently resistant to the contemporary environmental changes in the alpine grassland on the Tibetan Plateau. One challenge in our current study is that the activities of soil enzymes were assayed with a one-time sampling (August 2015, peak growing season and four years after treatment). They are potential activities (size of enzyme pools) that may not directly link to the treatment effects on the production, stabilization and turnover of soil enzymes *in situ* (Henry, 2012; Wallenstein and Weintraub, 2008). This challenge may further prevent us from a mechanistic understanding of soil enzymatic responses to environmental changes (Burns et al., 2013; Wallenstein and Weintraub, 2008). However, in recent years, the application of standardized microplate format assays (Deng et al., 2013; German et al., 2011) and the Michaelis-Menten kinetics of soil enzymes (German et al., 2012; Razavi et al., 2017; Stone et al., 2012), functional enzyme sequencing (Baraniya et al., 2016), *in situ* fluorogenic substrate addition technique (Steinweg, 2011) and soil zymography (Spohn et al., 2013) could be our next frontiers to resolve the challenge and provide mechanistic understanding of the responses of soil microbes to environmental changes in the alpine grasslands.

In summary, we combined two field experiments and a meta-analysis to examine the responses of soil enzyme activities to the main environmental changes in the alpine grasslands on the Tibetan Plateau. Our findings show that soil extracellular enzymes are insensitive to climate warming, altered precipitation and nutrient additions in these alpine grasslands even though effects on microbial biomass were observed. Therefore, the enzymatic adaptation to the alpine environment at the molecular level needs further investigation (e.g., low-temperature adaption, Saavedra et al., 2018). Given that the alpine grassland ecosystems are facing a number of natural and anthropogenic pressures such as climate change, N deposition, overgrazing and degradation (Chen et al., 2013), uncertainties remain in the responses of soil microbial community to these environmental changes. It is urgent for us to use cutting-edge enzymatic techniques to further investigate the effects of environmental changes on soil microbial functioning in the alpine grasslands on the Tibetan Plateau.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pedobi.2018.10.001.

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